

SOLUTIONS AND REAGENTS

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide)

STOCK SOLUTION

MTT 500 mg (Sigma M2128)
PBS 100 ml

Mix with vortex for 20 minutes, then sterile filter. Wrap bottle in aluminum foil or paper to block out light as MTT is light sensitive. Store at 4°C. Prepare fresh stock each month.

WORKING SOLUTION

MTT stock solution 10 ml
RPMI1640 + 20% FBS 40 ml

Prepare just prior to use and pre-heat to 37°C before use. To use add 1 ml of working solution to 2 ml of RPMI 1640/20% FBS containing the hollow fiber samples.

2.5% PROTAMINE SULFATE (P/S)

Protamine sulfate 2.5 gm (Sigma P4380)
normal saline (0.9%) 100 ml

Add P/S slowly to saline while stirring vigorously. Stir for 20 minutes or longer if particles are still present. Sterile filter and store at room temperature. Prepare fresh stock monthly.

NORMAL SALINE

(Quality Biological #14-106-5)

STERILE DISTILLED WATER

(Quality Biological #14-219-5)

RPMI 1640 w/o L-GLUTAMINE

(Quality Biological #12-113-5)

L-GLUTAMINE 200 mM

(Life Technologies/Gibco #320-5030)

FETAL BOVINE SERUM

(Hyclone Laboratories #A-1111-L)

TRYPSIN/EDTA

(Life Technologies/Gibco #610-5300)

PHOSPHATE BUFFERED SALINE

(Quality Biological)

70% ETHANOL (NON-DENATURED)

0.4% TRYPAN BLUE

TISSUE CULTURE

GENERAL

Cell lines (adherent and suspension) are split once or twice. Cells are fed once weekly in addition to splits.

EXPERIMENT PREP

Cells are split the previous week into T150 flasks at densities that will result in 70-90% confluency on the day of experiment. Cells are fed the day before the experiment by removing half of the existing volume of medium and replacing with an equal volume of fresh medium.

HOLLOW FIBER PREP

MATERIALS NEEDED

- hollow fibers (Spectrum Medical)
- sterile filtered deionized/distilled water
- chromatography columns
- large volume syringe with 20 gauge needle
- 70% EtOH (non-denatured)
- autoclavable pipette pan with cover (Nalgene)
- scissors
- RPMI 1640 with 20% FBS
- autoclave bags
- gloves (non-sterile)
- forceps

PROCEDURE

Wear gloves during all fiber manipulations to reduce transfer of body oils to fibers.

1. Using gloves, remove fibers from storage container and place them on work surface (previously wiped with EtOH to clean).
2. Position fibers so that badly crimped ends can be cut off and remaining lengths of fibers are even.
3. Cut the fibers into 12-15 inch lengths so they will fit into the pipette trays.
4. Pop open the ends of the tubing by gently squeezing across the crimp.
5. Flush the fibers with 70% EtOH individually using a syringe and 20 gauge needle and place them into a pipette tray containing 70% EtOH.
6. Transfer fibers to a chromatography column filled with 70% EtOH.
7. Store columns vertically at room temperature with fibers being allowed to soak a minimum of 72 hours (can be stored for weeks if necessary). Label column with the date processed.

→FROM THIS POINT ON DO NOT ALLOW THE FIBERS TO DRY OUT OR THE PROCESS HAS TO BE STARTED OVER AGAIN←

8. Pour the hollow fibers and alcohol in the columns into a pipette tray. Individually rinse the fibers with fresh 70% EtOH.
9. Fill a second pipette tray with 300-500 ml of filtered water to a pipette pan. Individually rinse the fibers with water and transfer into the water-filled pan.
10. Cover the pan and place into the autoclave. Autoclave at STP for 40 minutes (or whatever is required to change the sterility control pellet).

****FROM THIS POINT ON, ALL WORK WITH THE FIBERS MUST BE CONDUCTED UNDER STERILE CONDITIONS USING STERILE TECHNIQUE****

11. Place an empty, sterilized (autoclaved) pipette pan into the BSC. Fill the pan with 200-300 ml of RPMI 1640/20% FBS after removing the lid and placing it inside up onto a raised surface so that it can be picked up easily.
12. Sterily open 1 or 2 sterile fields in the hood.
13. Don sterile gloves and have assistant spray them with 70% EtOH.
14. Cover work area with sterile drapes and receive syringe and needle from assistant observing sterile technique.

HOLLOW FIBER PREP cont'd

15. Retrieve fibers from the pan of water and flush each fiber with RPMI 1640/20% FBS using a 20 gauge needle and syringe. Place the fibers into the pan containing the medium after individually flushing.
16. Fill pan with approximately 30 fibers, replace lid and tape both ends of lid so that it doesn't get dislodged. Label pan with date and prep conditions.
17. Spray outside of pan with 70% EtOH and place into a plastic bag. Loosely tape bag shut and place into the incubator.
18. Store at 37°C with 5% CO₂ until ready for use. Should be incubated at least overnight. Prolonged incubation does not appear harmful.

FIBER IMPLANT PREPARATION

MATERIALS NEEDED

- rocker plate
- 15 ml sterile conical centrifuge tube
- ice pack shaped around 15cc tube
- bacteriological incinerator
- sterile drapes (2)
- 10 cc syringe with 20ga catheter (chilled)
- 35 mm sterile plastic petri dishes
- 5 cc syringe with 20ga catheter (chilled)
- stainless steel sterile work surface
- sterile gloves
- sterile forceps (2 pair)
- sterile scissors
- smooth-jawed needle holders
- alcohol resistant markers (blue, green)
- conditioned hollow fibers

- Petri dishes should be labeled, filled with 2 ml of RPMI 1640/20% FBS and stored at 4°C until needed.
- Work surface should have 2 cm demarcations so that hollow fibers can be prepared at the proper length.
- Green and blue are the preferred marker colors because red and black produce very high background levels if inadvertently allowed to contaminate the sample at the time of MTT extraction.

PROCEDURE

→**CREATE AND MAINTAIN A STERILE FIELD THROUGHOUT THE PROCESS**←

1. Empty the BSC of all materials and wipe down with ethanol (including the interior glass). Put on a cuffed long sleeve gown/lab jacket.
2. Place the bacteriological incinerator, rocker plate and pipette tray containing the conditioned hollow fibers into the hood. Spray the external surface of the hollow fiber pan with 70% EtOH. Turn on the bacteriological incinerator so it can come to temperature.
3. Secure the Ascultured® ice pack to the rocker plate in the BSC.
4. Insert a sterile 15cc conical tip centrifuge tube into the ice pack. Remove the cap from the tube - do not touch the threads or opening of the tube as this tube will be used to hold the cell syringe so the Amouth® of the tube needs to be sterile to not contaminate the syringe.
5. Place the wrapped autoclaved work surface into the hood and unwrap being careful to not touch the tray or inside of the paper. Slide the tray to one end of the hood by grabbing the non-sterile end of the wrapping paper and pulling on it.
6. Unwrap all sterile instruments and drop them onto the work surface being careful to maintain sterility. Add the 10 cc and 5 cc syringes along with two 20 gauge catheters.
7. Sterily open the sterile drapes.
8. Don sterile gloves - **DO NOT TOUCH ANYTHING NONSTERILE FROM THIS POINT ON.**
9. Have gloved hands sprayed with 70% ethanol by assistant to remove/restrict glove powder.
10. Sit down and pull up to the hood.
11. Unfold 1 sterile drape and spread along the width of the hood surface being careful not to touch the upper working surface side to anything nonsterile.
12. Unfold second drape and spread parallel to and slightly overlapping the first drape.
13. Place stainless steel work surface onto the sterile field drapes. Assistant discards the paper wrapping from the sterile work surface.
14. Gloved (nonsterile) assistant removes lid from the pan of hollow fibers maintaining sterility of the inner lid surface.
15. Assistant opens tube of cold media under the hood and holds while the 10 cc syringe is filled. Media filled syringe is placed to one side while assistant removes the tube.

FIBER IMPLANT PREPARATION cont-d

16. Assistant holds tube containing the cell suspension in the hood while the 5 cc syringe is filled with cell suspension. A 5 cc syringe is used for the cell suspension because it is the size syringe that will fit nicely into the 15 cc conical tube which is located in the ice pack.
17. Place the cell syringe into the 15 cc conical tip tube located in the ice pack so the cells remain cold and mixed. DO NOT TOUCH THE TUBE EXTERIOR or THE ICE PACK- unsterile!!! It is important to keep the cells cold to reduce agglutination and to increase viability.
18. Using sterile forceps remove 3-6 fibers from the sterile pan and place them onto the work surface where they are flushed with fresh medium.
19. Remove cell syringe from holder and aspirate a small volume of air.
20. Slide the hollow fiber over the catheter until the bevel is covered. Inject a small volume of air through the fiber to remove any residual medium then fill with the cell suspension being careful to exclude air bubbles.
21. Place the needleholder into the incinerator and heat for a few seconds (generally 3-5 seconds is adequate). Heat seal the loose end of the hollow fiber by briefly clamping the end with the needleholder. Heat seal the end of the fiber attached to the catheter and lay the fiber down.
22. Return the cell syringe to the ice holder.
23. Using the medium filled syringe cover the exterior of the fibers with medium to prevent them from drying out. 3-6 fibers can be processed at a time but care must be taken to prevent the fibers from drying out or the medium osmolality from increasing to a deleterious level.
24. Using the 2 cm marks on the work surface to indicate distance, heat seal every 2 cm. Cut the individual samples apart in the center of the heat seal. The heat seals should be clear, not white, in appearance. The desired length of a heat seal is 3-4 mm so that when the samples are cut apart each sample has a 1.5 - 2 mm long sealed "flap". Generally, the first heating marks the point that should be sealed and a second heating is done while exerting a slight pull across the seal to produce the actual heat seal. If necessary, the seal can be reheated until the desired effect is achieved. PVDF heat seals well when wet or when dry. For the purpose of cell samples it is important that the fiber not be allowed to dry.
25. Assistant places 35 mm dishes into the BSC and removes the lid so that 6-12 fibers can be transferred into each dish for storage. Store all fibers at 4°C until the entire cell line has been prepped.
26. Repeat steps 18-25 until the proper number of samples have been generated.
27. Implant fibers into host mice (SCIDs generally) as soon as possible after preparation.

****When using more than one cell line for an experiment, implants must be color-coded to differentiate cell lines following in vivo implantation. To color code: before heating the needle holders, saturate the inner surface of the jaws with the desired color by squeezing the tip of the marker several times.****

NOTES ON FIBER IMPLANT PREPARATION

1. VISUALLY CHECK THE CELLS IN THE SYRINGE TO DETERMINE WHETHER THEY HAVE AGGLUTINATED OR AGGREGATED WHILE SITTING. IF THEY HAVE THEN ATTEMPT TO DISAGGREGATE BY GENTLY MIXING. IF THEY DISAGGREGATE USE THEM. IF THEY REMAIN AGGLUTINATED THEN NEW CELLS SHOULD BE GOTTEN. FOR SOME CELL LINES IT IS VERY PROBABLE THAT THEY WILL AGGLUTINATE/AGGREGATE - FOR SOME OF THESE LINES IT IS BEST THAT THEY BE HELD IN A CENTRIFUGE TUBE IN ICE RATHER THAN IN THE SYRINGE AS THEY STAY COLDER AND THUS LESS LIKELY TO AGGREGATE.
2. SERUM PROTEINS BEGIN TO COOK ONTO THE NEEDLEHOLDERS AS THEY ARE USED. THIS INTERFERES WITH EFFICIENT HEAT SEALING. THE FORCEPS CAN BE CLEANED BY SCRAPING WITH THE BLADE OF THE SCISSORS OR BY WIPING WITH AN ETHANOL IMPREGNATED SWAB. IF ETHANOL IS USED THE FORCEPS SHOULD BE HEATED TO REMOVE THE ALCOHOL PRIOR TO COMING IN CONTACT WITH CELL SAMPLES.
3. DO NOT SQUEEZE FIBERS EXCESSIVELY, ESPECIALLY WITH THE FORCEPS DURING THE PROCESSING. THE SITES WHERE SQUEEZING OCCURRED ARE MORE OFTEN THAN NOT DEVOID OF ANY CELL GROWTH.
4. FIBERS WHICH DEHYDRATE (TURN WHITE) SHOULD NOT BE USED. IF THEY HAVE NOT YET BEEN EXPOSED TO CELL SUSPENSION THEN THEY CAN POSSIBLY BE REPROCESSED.
5. IF FIBERS CONTAIN A FLATTENED OR BENT PLACE THEN HEAT SEALS SHOULD BE PLACED SO THE DAMAGED AREA CAN BE REMOVED.
6. IF FIBER SAMPLES TURN WHITE THEN THEY ARE UNACCEPTABLE FOR USE.
7. TEMPERATURE FLUCTUATIONS SHOULD BE AVOIDED AS MUCH AS POSSIBLE AS THEY ARE VERY DELETERIOUS TO THE CELLS.

ASSAYING IMPLANTS

COLLECTION

1. Fill collection vessels (35 mm dishes or sterile specimen cups) an adequate volume of RPMI 1640/20% FBS and place into incubator. Transfer vessels to *in vivo* laboratory for sample collection.
2. Sacrifice mice (3 maximum at any given time). Collect subcutaneous fibers into SC labeled dish. Collect intraperitoneal fibers into IP labeled dish.
3. Return dishes containing fibers to incubator as soon as possible.

**** Maintaining temperatures at or near 37°C is very important to retaining cell viability.**

4. Transfer dishes from *in vivo* laboratory to *in vitro* laboratory. Minimize temperature fluctuations as much as possible.

PROCEDURE (Alley et al. 1991 Cancer Res 51: 1247-1256).

1. Transfer fibers for MTT assay into individual wells of 12 well plates containing 2 ml of RPMI 1640 with 15% FBS, 2mM L-glutamine, 50 ug/ml gentamycin and 50 U/ml penicillin.
2. Incubate plates for 30 minutes in a humid 5% CO₂ atmosphere at 37°C.
3. Add 1 ml of MTT working solution (pre-warmed to 37°C) and incubate for 4 hours at 37°C in a 5% CO₂ atmosphere.
4. Transfer each fiber to a 1 ml titertube containing 1 ml of 2.5% protamine sulfate (PMS) solution.
5. Store at 4°C for 24 hours.
6. Aspirate protamine sulfate and add 1ml of protamine sulfate for a second wash.
7. Store at 4°C for at least 4 hours. The samples can be stored for > 2 weeks which allows an entire experiment to be batch tested.
8. Wipe each fiber with gauze to remove any stained debris from the exterior of the fiber, transfer to a 400µl microtest tube and air dry for 24 hrs.
9. Add 250µl of DMSO (Burdick & Jackson) and incubate at room temperature for 4 hrs protected from light (MTT is light sensitive).
10. Transfer 150 µl of each sample to 96-well plates and read the plates at 540-570 nm in a standard microtiter plate reader.

FIBER PREPARATION FOR RT AND p24 Ag ASSAYS

1. Randomly separate the fiber samples following collection into groups for MTT assay and for RT and p24 antigen assay.
2. Place each fiber to be assayed for RT activity and/or p24 antigen into a 1.5 ml Eppendorf safe lock tube and store at -20°C until all fiber collection timepoints are complete so all the fibers for a single experiment can be batch tested.
3. To assay, thaw the Eppendorf tubes and add 250 µl of lysing buffer (0.02 M Tris-HCl (pH 7.5), 0.05 M NaCl, 0.5% Triton X-100) to each tube.
4. Cut the fibers in the tubes using curved tip scissors.
5. Incubate at room temperature for 30 minutes.
6. Transfer 150 µl of the lysate into the well of a 96 well round bottom microtiter plate. This serves as the source of sample for the RT and p24 antigen assays.

RT ASSAY PROCEDURE

MATERIALS

tritiated thymidine triphosphate (specific activity: 2.5 mCi/ml in 1.5 M Tris buffer (NEN)
rAdT stock [0.5 mg/ml poly rA (Pharmacia) and 1.6 units/ml oligo dT (Pharmacia)]

5X RT reaction mix (prepare fresh daily):

125 µl 1M EGTA

125 µl dH₂O

125 µl 20% Triton X-100

50 µl 1M Tris (pH 7.4)

50 µl 1 M DTT

40 µl 1 M MgCl₂

Whatman DE 81 filter paper

PROCEDURE

1. Prepare the complete RT reaction mixture by mixing 1 part tritiated thymidine, 2.5 parts rAdT, 2.5 parts 5X reaction mix and 4 parts water.
2. Place 10 µl complete RT reaction mixture into each well of a 96 well round bottom microtiter plate.
3. Add 15 µl of fiber lysate to the test wells and mix.
4. Incubate samples for 1 hr at 37°C.
5. Spot sample onto DE81 filter paper.
6. Wash filters 6 times (5 minutes each) in 5% sodium phosphate buffer.
7. Wash filters 2 times (1 minute each) in distilled water.
8. Wash filters 2 times (1 minute each) in 95% EtOH.
9. Place filters into plastic scintillation vials and dry.
10. Add 3 ml of Opti-Fluor O (Packard) to each vial and quantitate the radioactivity in a liquid scintillation counter (Packard Tri Carb 1900 TR).

p24 ANTIGEN ASSAY

The p24 antigen assay is conducted following the manufacturer's protocol. The studies herein were conducted using a murine monoclonal antibody based assay kit purchased from Coulter Corporation (Hialeah, FL).